Encoding regulatory state boundaries in the pregastrular oral ectoderm of the sea urchin embryo

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By gastrulation the ectodermal territories of the sea urchin embryo have developed an unexpectedly complex spatial pattern of sharply bounded regulatory states, organized orthogonally with respect to the animal/vegetal and oral/aboral axes of the embryo. Although much is known of the gene regulatory network (GRN) linkages that generate these regulatory states, the principles by which the boundaries between them are positioned and maintained have remained undiscovered. Here we determine the encoded genomic logic responsible for the boundaries of the oral aspect of the embryo that separate endoderm from ectoderm and ectoderm from neurogenic apical plate and that delineate the several further subdivisions into which the oral ectoderm per se is partitioned. Comprehensive regulatory state maps, including all spatially expressed oral ectoderm regulatory genes, were established. The circuitry at each boundary deploys specific repressors of regulatory states across the boundary, identified in this work, plus activation by broadly expressed positive regulators. These network linkages are integrated with previously established interactions on the oral/aboral axis to generate a GRN model encompassing the 2D organization of the regulatory state pattern in the pregastrular oral ectoderm of the embryo.

regulatory state boundaries | pattern formation | repression circuitry

By the onset of gastrulation, bilaterian embryos consist of a complex mosaic of sharply bounded regulatory state domains, where "regulatory state" refers to the sum of specifically expressed mRNAs encoding DNA sequence-recognizing transcription factors in each nucleus. The regulatory state domains or territories are organized spatially in respect to the two major axes of the embryo, and they constitute informational specifications that determine the subsequent embryonic fates and functions of the cells descendant from these domains. Although in different modes of pregastrular embryogenesis regional specification functions are accomplished in somewhat different ways (1, 2), the end result is always the same: subdivision of the embryo into (transient) spatial regulatory states. These progressively specified regulatory states, and the boundaries between them, are the output of networks of genomically ordained interactions among regulatory genes. Gene regulatory networks (GRNs) encompass the heritable code for the embryonic development of each species. At present, the best known, experimentally determined, large-scale GRN drives the specification of endoderm and mesoderm in the embryo of the sea urchin *Strongylocentrotus purpuratus* up to gastrulation (3–6). This GRN model encompasses about half of the embryo, covers 30 h of development (18 h in the nonskeletogenic mesoderm), and all or almost all relevant regionally expressed regulatory genes. A recent study (7) shows that the endomesoderm GRN model contains sufficient regulatory relationships to generate a computational automaton that successfully predicts almost all spatial and temporal regulatory gene expression in this phase of embryogenesis.

Encompassing the whole of the pregastrular embryo in an approximately complete, causal, GRN model such as that constructed for the endomesoderm, is now a conceivable objective. The major territories remaining to be considered at this level are the oral and aboral ectoderm and the neurogenic ciliated band and apical domains, as well as the later oral and aboral mesoderm. Major progress on the GRN linkages within all these regions except the apical domain has recently been attained (8–13). Regulatory state patterns within them are organized in an essentially orthogonal manner with respect to both the animal/vegetal axis and the oral/aboral axis of the embryo. Here we take up the problem of understanding the mechanistic nature of the control system that specifically sets the boundaries of the increasingly complex regulatory state domains along these axes in the oral ectoderm. More exactly, this work enhances extant oral ectoderm GRN models by defining the system of regulatory relationships by which are set the boundaries between the oral ectoderm, the apical domain, and the endoderm, as well as the boundaries between those regulatory states arising within the oral ectoderm. We have successfully used a simplifying strategy, based on the idea that the initial regulatory gene expression domains that first respect a given territorial boundary are the outcome of the regulatory interactions defining that boundary, and thus these genes have become the preferred targets of perturbation analyses. This strategy requires a priori a relatively complete knowledge of the dynamic spatial and temporal regulatory gene expression patterns within the oral ectoderm, and we begin with a summary of these patterns.

Results

Oral Ectoderm Regulatory States from Cleavage to Gastrulation. As shown in Fig. S1*A*, cohorts of regulatory genes are expressed in increasingly complex patterns as development proceeds. The process of regulatory state formation is summarized in diagrams (Fig. 1), which are based on single and double in situ hybridization from earlier studies of regulatory gene expression (5, 8–11, 14, 15) and from detailed additional observations that we reproduce in Fig. S1. Among 35 oral ectoderm and apical reg-

Significance

Regulatory state boundary formation is a general process in early development, in which embryonic territory is divided up into spatial domains that express distinct sets of regulatory genes. We establish the mechanistic principles by which multiple orthogonal boundaries of this kind are progressively formed on the oral side of the sea urchin embryo, according to an encoded genomic program. These boundaries separate prospective endoderm from ectoderm domains, neurogenic from non-neurogenic domains, and ciliated band from oral ectoderm domains and produce an orthogonal grid of regulatory states. Boundary formation invariably depends on spatial transcriptional repression superimposed on more widespread domains of transcriptional activation.

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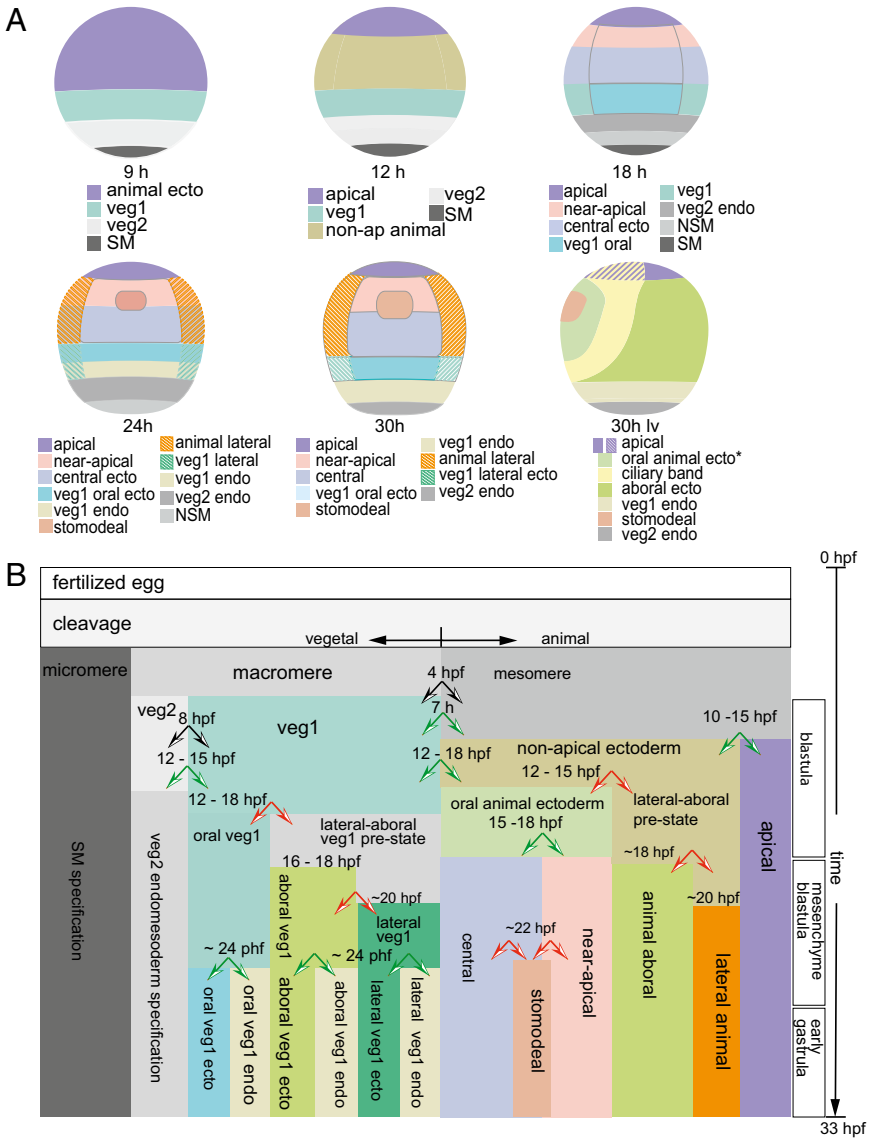


Fig. 1. Diagram of ectodermal regulatory states with increasing complexity from the late cleavage stage to the onset of gastrulation. (*A*) Maps showing regulatory state formation. Developmental stages include the late cleavage stage (9 h), blastula stage (12, 15, and 18 h), mesenchyme blastula stage (24 h), and early gastrula stage (30 h). The embryos are shown with oral ectodermal views unless specified otherwise. lv, lateral view. The asterisk (*) following "oral animal ecto" indicates near-apical + central ectoderm. (*B*) Events of cell-state divergence during early embryogenesis, highlighting ectoderm diversification. Red orthogonal arrows indicate separation of regulatory states along the oral-aboral axis, and green arrows indicate the animal-vegetal axis; black orthogonal arrows indicate cell lineage separation.

ulatory genes previously reported, 12, generally the earliest markers of each individual regulatory state domain, were selected for further examination. These genes play critical roles in regulatory state formation and diversification. Their quantitative expression dynamics had been measured earlier (16), and the relevant kinetic data are reproduced in Fig. S2. The detailed sequence of expression patterns shown in Fig. S1 can be abstracted to provide the dynamically changing Boolean expression matrices shown in Fig. 2, where the contributions of the 12 genes to the regulatory state of each domain can be read horizontally (6, 7). These are the specific patterns of expression for which we seek causal explanation in the encoded architecture of the ectodermal GRNs.

The ectodermal boundary formation events in both time and space that are implied in the patterns of Fig. 1*A* are abstracted in the regulatory process diagram shown in Fig. 1*B*, where the orthogonal pairs of arrows mark the institution of regulatory state boundaries—red for boundaries in the oral/aboral axis and green for boundaries that arise along the animal/vegetal axis. These boundaries are the subject of the experimental work in this paper (except for one arising within the aboral ectoderm, the bottom-right image in Fig. 1*A*). An initial transient regulatory state boundary, already evident at 7 h, forms at the equatorial cleavage planes where it separates *eve*-expressing blastomeres that will give rise to endomesodermal constituents (macromere descendants) from *foxq2*-expressing blastomeres ancestral only to ectodermal and neurogenic components of the embryo (mesomere descendants) (Fig. S1, 7 and 9 h). A new regulatory state boundary is then established, which separates the nonapical ectoderm from the apical domain (10–15 h). A boundary between the transient regulatory states of all *veg1* lineage cells and that of the overlying oral ectoderm forms at 12–18 h; at 15–18 h, another regulatory state boundary delimits the near-apical from the central-oral ectoderm regulatory states; and finally, at ~24 h, the

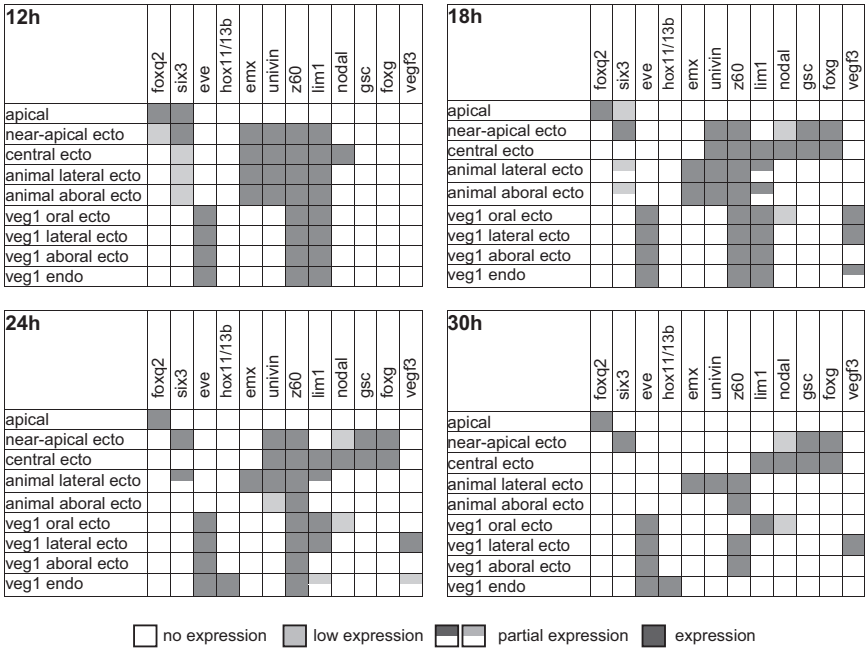


Fig. 2. Expression matrix of ectodermal regulatory genes. Expression territories of the regulatory genes were mapped using WMISH (Fig. S1) and summarized in the matrix table. All domains and subdomains refer to the ectoderm unless otherwise specified. The matrix table includes only regulatory states on the oral or the lateral side of the embryo. Developmental stages include 12 h (early blastular stage), 18 h (late blastular stage), 24 h (mesenchyme blastular stage), and 30 h (early gastrula stage).

definitive regulatory state boundary separating *veg1* posterior endoderm from *veg1* ectoderm is established. Boundaries within the apical neurogenic domain have not been analyzed, whereas formation of boundaries within the endomesoderm was solved previously (5, 6). We adduce earlier evidence regarding oral ectoderm regulatory state boundaries that form along the oral/aboral axis and integrate it with the animal/vegetal boundary mechanisms below.

GRN Interactions Controlling the Apical Domain/Oral Ectoderm Boundary. The mechanism leading to specification of the apical neurogenic domain begins with the zygotic expression of *foxq2*, which, as we have just seen, is activated during the fifth cleavage in all cells of the animal hemisphere (Figs. S1 and S2). A sharp upward retraction in the domain of *foxq2* expression occurs between 9 and 12 h, however, and this process continues, so that by 15 h the spatial expression of *foxq2* marks the apical plate region; thereafter, this gene is stably expressed in the central region of the apical plate domain (Fig. S1, 9–30 h). Our initial objective, in unraveling the gene interactions that set the boundary between the apical domain and the oral ectoderm, is to understand the cause(s) of retraction of the *foxq2* expression domain until it arrives at and defines the apical plate boundary because it is the first apical-specific regulatory gene to be expressed. Some evidence indicated that the earliest 9- to 12-h phase of retraction could be mediated indirectly by Wnt8 signaling from the vegetal blastomeres, and indeed effects of Wnt signaling on the size of the apical domain were reported earlier (17). We show here, however, that after 12 h a known transcriptional repressor expressed in the oral ectoderm, *not*, which, however, is not a direct target of Wnt signaling, is specifically responsible for preventing *foxq2* expression all the way down to the equator. This is demonstrated by the dramatic spatial effects of *not* morpholino antisense oligonucleotide (MASO) on *foxq2* expression at 15 h, which, as seen in Fig. 3*A*, is then restored down to the oral equator. Fig. 3*A* also shows that this MASO effect cannot be seen 3 h earlier. The *not* gene, a direct early target of Nodal signaling (10), is expressed significantly in the equatorial oral ectoderm by 12 h and thereafter (Fig. S2).

Fig. S1 also shows that transcripts of the homeobox gene *emx*, the zinc-finger gene *egr*/*z60* (18), and the Tgfβ gene *univin* abut the lower boundary of *foxq2* expression at 15 and 18 h and that all three are transcribed in the regions from which *foxq2* has earlier cleared. This is confirmed in the control *emx*/*foxq2* double in situ hybridization shown in Fig. 3*A*, and in Fig. S3*A* the same is shown for *univin* and *z60*. The *emx* gene repressively controls the lower boundary of *foxq2* expression after the midblastula stage, as shown by the spread of this domain into the near-apical oral ectoderm in *emx* MASO-treated embryos (Fig. 3*A*). Reciprocally, as Fig. 3*A* shows, *foxq2* expression is required to prevent expression within the apical domain of *emx* (and also of *univin* and *egr*) (Fig. S3*A*). Thus, *emx* and *foxq2* are linked in a mutual exclusion circuit (4, 19, 20), in which, within the normal domain of expression of each, expression of the other is prohibited. Thereby, the boundary separating *foxq2* expression from the oral ectoderm regulatory state is locked down. The logical elegance of this boundary formation mechanism is underscored by the use of a common driver by both *foxq2* and *emx* genes. This is the positively acting, pan-ectodermal regulator SoxB1 (21): as shown by the quantitative MASO perturbation experiments reproduced in Fig. S4, by 24 h *emx* and *foxq2* expression are, respectively, about 90 and 97% depressed by knockdown of SoxB1 translation. Note that, later in blastulation, expression of *emx*, *univin*, and *egr* is cleared from the oral and/or aboral ectoderm (Fig. S1*A*) by repressors active in that domain, whereas their boundaries with *foxq2* persist on the oral side of the embryo.

Gene Interactions Controlling the Boundary Between Animal and Vegetal Ectoderm. All cells deriving from the animal half of the sea urchin embryo give rise to ectoderm, but, in addition, a few cells of the vegetal half located immediately beneath the equator also assume ectodermal cell fates. These cells are descendants of the *veg1* lineage, which also gives rise to posterior endoderm. In the ancestors of *veg1* ectodermal cells, *eve* expression initiates at

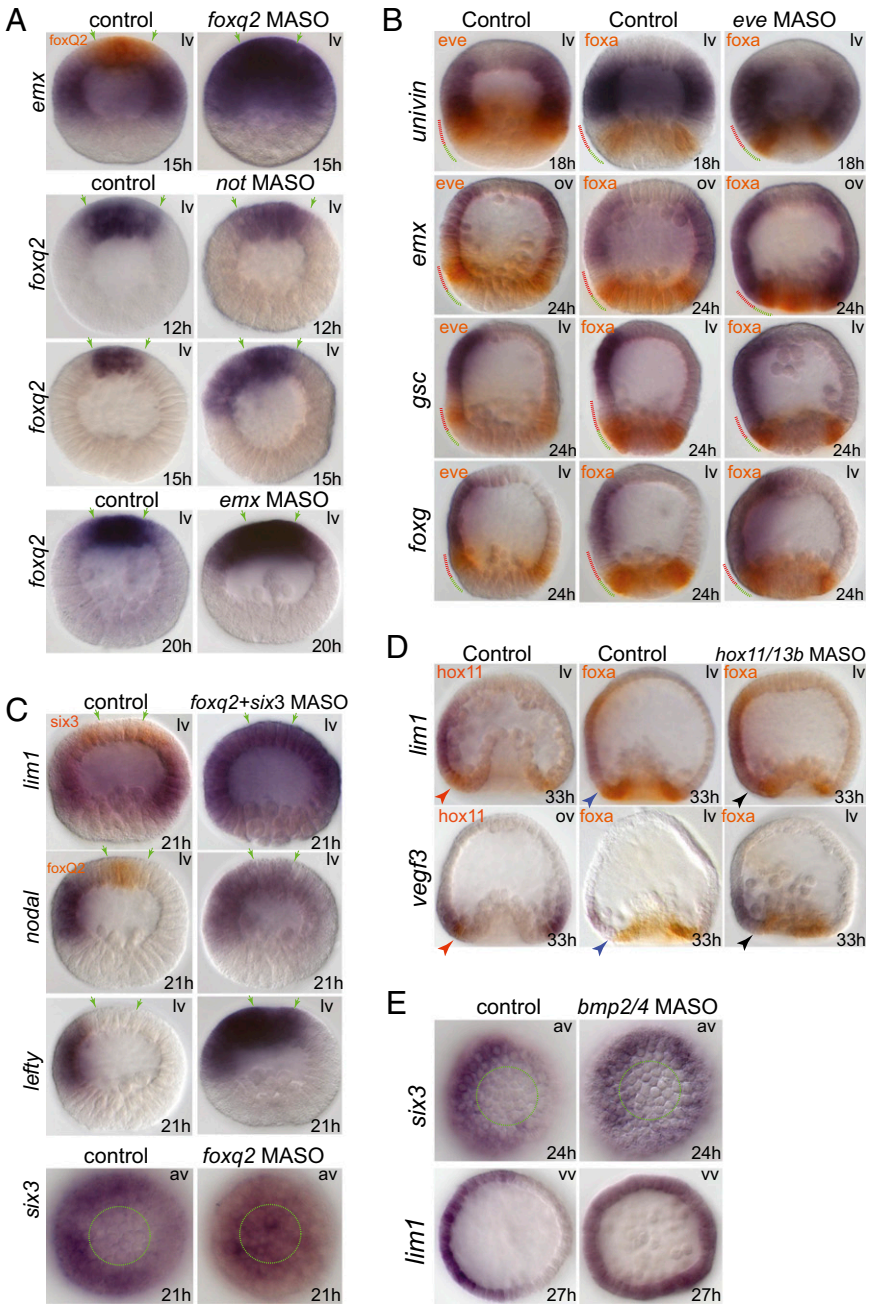


Fig. 3. MASO perturbation and expression analysis to identify the roles of regulatory genes in establishing boundaries along the primary axis. Perturbation assays were performed to knock down expression of *foxq2*, *not*, or *emx* (*A*), *eve* (*B*), *six3* and/or *foxq2* (*C*), *hox11/13b* (*D*), or *bmp2/4* (*E*). Expression pattern changes were investigated using WMISH. The apical region of endogenous *foxq2* expression is marked with green arrowheads or dotted circles (*A*, *C*, and *E*). Red and green dashed arcs in *B* indicate the *veg1* and *veg2* regions, respectively. In *D*, the red arrowheads mark *hox11/13b* expression; the blue arrowheads indicate the gap between the *veg1* ectoderm and the *veg2* endoderm, and the black arrowheads show expansion of *lim1/vegf3* expression into the *veg1* endoderm in the *hox11/13b* morphants. A broader survey of more regulatory genes in response to MASO perturbation was shown in Fig. S3. lv, lateral view; vv, vegetal view; av, apical view; ov, oral ectodermal view.

7 h, representing the first regulatory distinction from animal ectodermal cells (5, 6, 22). Until about 12 h, *eve* remains broadly expressed in the vegetal half, but by 15 h its expression is confined to the *veg1* lineage, and transcript levels accumulate (Fig. S2) (5). Expression of *eve* precedes expression of the genes constituting the regulatory state of the adjacent nonapical ectoderm such as *emx* and *lim1* (Figs. S1*A* and S2). When *emx* is activated, and until after 15 h, it is expressed all over the nonapical ectoderm, and in addition its lower boundary of expression overlaps with that of *eve*. The *univin* gene is expressed in exactly the same way (Fig. S1*A*). After 18 h, however, transcripts of both genes have cleared from the *veg1 eve* domain (*emx* is then expressed in the lateral and aboral ectoderm). This behavior suggests that *eve* is acting as a repressor of genes that define the animal ectoderm regulatory states on both the oral and the aboral side of the embryo.

The experiments of Fig. 3*B* demonstrate this *eve* function. In the first column, we see double in situ hybridizations showing *eve* and *emx* and *eve* and *univin*. Expression of *univin* still partly overlaps

that of *eve* at 18 h, but its expression and that of *emx* have become exclusive with respect to *eve* on either side of the *veg1*/animal ectoderm boundary by 24 h. Two additional genes expressed in the oral ectoderm GRN, *gsc* and *foxG* (9, 10), are also expressed right down to the boundary with the *veg1 eve* expression domain at 24 h. The second column of Fig. 3*B* shows expression of these same four ectoderm genes together with that of the veg2 regulatory gene *foxa*: a gap of three to four rows of cells deep, which corresponds to the *veg1* domain, is clearly visible for all four genes. The third column demonstrates for each of these genes that, if *eve* expression is blocked by introduction of MASO, their expression extends right down to the veg2 cells, and the *veg1* gap no longer exists. Therefore, *eve*, which defines *veg1* regulatory identity and initiates the *veg1* GRN (6), also functions to exclude expression of these ectoderm genes from the *veg1* domain. Thereby, the *veg1* lineage boundary is maintained as a regulatory state boundary, i.e., until the following boundary transition in this region, which subdivides *veg1* into posterior endoderm and ectoderm.

Transcriptional Repressions Further Partitioning the Oral Ectoderm. The *lim1* regulatory gene is activated by ~10 h, 2–3 h after *foxq2* and *six3* (Fig. S2). Its expression is also driven by the pan-ectodermal activator SoxB1 (Fig. S4). A priori this gene should therefore be able to express throughout the ectoderm, but, instead, from the outset its expression appears as a band extending around the embryo from the *veg1* domain below the equator up to about halfway into the animal oral ectoderm (Fig. S1*B*, 12–21 h). Its upper boundary of expression implies repression of *lim1* in the apical and near-apical animal ectoderm. At 15 and 18 h, the expression pattern of *six3* (15, 23) appears perfectly reciprocal to that of *lim1*. Thus, as seen in lateral view, *six3* transcripts occupy the apical domain plus the near-apical animal ectodermal domains (Fig. S1*B*). An experiment shown in Fig. 3*C* demonstrates that *six3* and *foxq2* are indeed responsible for excluding *lim1* expression from the apical and near-apical ectoderm because *foxq2* + *six3* MASOs cause *lim1* expression to extend to the whole animal half of the embryo. The central animal ectoderm and the near-apical animal ectoderm regulatory states are thereby separated by the *lim1*/*six3* boundary. This boundary soon pertains only to the oral side, where it persists, as *lim1* expression is lost from the aboral ectoderm between 21 and 24 h, as is *six3* expression (Fig. S1*B*). Furthermore, an additional spatial repression mediated by *foxq2* precludes *six3* expression in the central apical domain (Fig. 3*C*). Thus, the relations *foxq2* repressing *six3*; *foxq2*+*six3* repressing *lim1* produce a central disk of *foxq2* expression; a surrounding torus of *six3* expression, the lower boundary of which bisects the nonapical animal ectoderm; and an abutting lower torus of *lim1* expression. Following the confinement of *lim1* and *six3* to the oral side of the embryo by aboral ectoderm repression (see below), these boundaries persist in the oral ectoderm and oral apical domains (Fig. S1*B*).

Exclusion of the Oral Ectoderm GRN from the Apical Domain. Repression by *foxq2* plus *six3* has a further spatially specific effect, the ultimate significance of which expands as early development proceeds. This is the repression of *nodal* expression. Although the repressive role of *foxq2* was previously proposed (14), the regulatory circuit governing the dynamic *nodal* expression is more complicated and involves synergetic repression. As summarized in Fig. 2, the *nodal* gene is expressed strongly in the central oral ectoderm and more weakly in the near apical oral ectoderm (see *foxq2*/*nodal* double in situ hybridizations in Fig. S1*C*). In the absence of [*foxq2*+*six3*] expression, *nodal* transcription spreads upward over the whole oral apical domain (Fig. 3*C*), although treatment with either *foxq2* or *six3* MASO alone has only minor effects (Fig. S3). This observation, at 21 h, suggests that persisting Six3 protein is responsible together with Foxq2 protein for apical *nodal* repression (by 21 h, *six3* is no longer being transcribed in the apical domain; Fig. 3*C*). Transcriptional target genes of Nodal signaling such as *lefty* are, as expected, affected by [*foxq2*+*six3*] MASOs in exactly the same way as is *nodal* expression (Fig. 3*C*). Partial repression by Six3 probably accounts for the relatively weak *nodal* expression in the near-apical animal oral ectoderm. Expression of the *nodal* gene is the primary transcriptional response to the redox polarization that in causal terms initially generates the future oral/aboral axis (24–28). Therefore, because much of the oral ectoderm-specific GRN is wired downstream of Nodal response genes (9–11, 29, 30), Foxq2 repression of *nodal* hierarchically confines the whole oral ectoderm GRN to the region below the *foxq2* expression boundary, that is, the "apical/near apical" boundary of Fig. 1 *A* and *B*.

Transcriptional Repression Defining the Boundary Between Ectoderm and Endoderm. The last of the pregastrular boundaries formulated on the animal/vegetal axis to be considered here is that separating all ectoderm fates from endodermal fates. This boundary forms within the *veg1* cell lineage, which gives rise to posterior endoderm and to ectodermal cells located just below the equator (31, 32). Directly or indirectly, Wnt5 signaling is involved in initial *veg1* specification (33). Further separation of the *veg1* regulatory state, and ultimately of embryonic fate, occurs after 24 h in the late mesenchyme blastula stage (Fig. 1*B*). By the end of gastrulation, *veg1*-derived endoderm has constituted the hindgut and part of the midgut, whereas *veg1*-derived ectoderm has formed the wall of the embryo surrounding the anus. The first spatial regulatory state changes denoting formation of this boundary are separation of the expression domains of *lim1* and *vegf3* from that of *hox11/13b* (Fig. 2). Up to 24 h, *lim1* and *vegf3* are transcribed in all *veg1* cells, including the future endoderm precursors; thus the expression domains of these genes abut that of the *veg2* gene *foxa* as can be seen in the double in situ hybridizations of Fig. S1*B* (*foxa*/*lim1*) and Fig. S1*C* (*foxa*/*vegf3*). By 24 h, *hox11/13b* expression is initiated in the lower rings of *veg1* cells, defining those destined for endodermal fate (6). Thereafter, *lim1* and *vegf3* cease to be expressed in these presumptive endoderm cells, and, by 30 h, the *veg1* endoderm below the newly formed endoderm/ectoderm boundary expresses *hox11/13b* and not *lim1* or *vegf3*, whereas the *veg1* oral ectoderm cells do not express *hox11/13b* but do express *lim1*, and those oral ectoderm cells immediately lateral to the *lim1*-positive cells express *vegf3*. Put more generally, *veg1* cells expressing *hox11/13b* become endoderm and *veg1* cells not expressing *hox11/13b* become ectoderm. Because the *lim1* and *vegf3* genes are expressed only a few hours earlier coincidentally with *hox11/13b*, a reasonable prediction is that, when *hox11/13b* is transcribed in the lower *veg1* cells, this gene establishes the endoderm/ectoderm boundary by repressing ectoderm-specific genes within its domain of expression.

Fig. 3*D* shows that, if *hox11/13b* expression is blocked by MASO treatment, clearance of neither *lim1* nor *vegf3* expression from prospective posterior endoderm cells fails to occur. This is demonstrated by the extension of the domains of expression of these genes right to the veg2 boundary of *foxa* expression. Thus, *hox11/13b* acts as a critical domain-specific repressor on the endoderm side of the boundary separating endodermal from ectodermal cell fate in the sea urchin embryo.

Lateral Boundaries. Fig. 1*A* illustrates two bilateral boundaries on the oral face of the 30-h embryo that separate regulatory states along the oral/aboral axis. These are the boundaries on each side between the animal lateral ectoderm and the medial ectoderm territories, i.e., the near-apical and central-oral ectoderm and, within the *veg1* ectoderm, the boundaries on each side that separate the lateral *veg1* ectoderm from the central *veg1* oral ectoderm (in addition, there are the boundaries of the future stomodaeum, which are not treated here). It is to be noted that appearances can be deceiving, so to speak, in that the regulatory state map of the

embryo is significantly more complex than the morphological map of the oral side of the embryo would suggest. Thus, as shown by comparing the 30-h lateral and oral views of Fig. 1*A*, the ciliated band actually consists of the two bilateral ectoderm regulatory states—an oral apical regulatory state and the central and lateral *veg1* regulatory states—even though the regulatory gene *one-cut* (*hnf6*) is expressed early all around the future ciliated band.

The regulatory states of the aboral ectoderm domains (11, 12) differ from those of the oral ectoderm domains, although some key genes contributing to each are at first expressed in both oral and aboral ectoderm. We have already encountered two examples, *six3* and *lim1* (Fig. S1*B*). Resolution of their expression patterns to the oral side again depends on repression. Many genes of the aboral ectoderm require a positive boost from Bmp signals emitted from the oral ectoderm, as confirmed at the *cis*-regulatory level (12). Because of the extensive feedbacks within the aboral ectoderm GRN, Bmp MASO essentially down-regulates the whole of this GRN. As shown in Fig. 3*E*, use of Bmp MASO demonstrates that the aboral ectoderm GRN includes repressor(s) that function to abolish transcription of *six3* and *lim1* in the aboral ectoderm.

Returning to the two pairs of oral/aboral boundaries within the oral face, much has been learned about the specific repressions responsible for the boundaries between the medial and lateral ectodermal territories. Two known genes, *not* and *gsc*, encode spatial repressors that are expressed in the oral ectoderm, both activated by Nodal signaling. The *not* gene is expressed in the near apical, central, and *veg1* oral ectoderm by 15 h, and *gsc* is expressed in the same domains except for the *veg1* oral ectoderm. Repression by *not* silences multiple genes of the lateral oral ectoderm regulatory state in the medial oral ectoderm domains, leaving them to be expressed across the boundary with the lateral oral ectoderm (9, 10). For genes initially expressed across the *veg1* ectoderm such as *vegf3*, *not* repression confines expression to the regions across the boundary with the *veg1* lateral ectoderm (10). Repression by *gsc* silences the *onecut* gene in the near apical and central oral ectoderm, confining its expression to the ciliated band domain across the boundaries with the lateral ectoderm on the sides, the apical domain above, and the *veg1* ectoderm that constitutes the oral/vegetal arm of the ciliated band (34). Aboral repressors such as *irxa* restrict the expression of all of the genes of the lateral oral and *veg1* oral ectoderm to confined bands of cells, also obliterating their expression in the aboral ectoderm (34).

Thus, an essentially orthogonal, bilateral pattern of bounded oral ectodermal regulatory states is established by the time of gastrulation (Fig. 1*A*, 30 h). The observations that we summarize in this paper, taken together with those obtained earlier, show that the mechanism by which this complex pattern is established is mainly sequential, spatially confined, transcriptional repression, occurring along both axes of the embryo.

Discussion

Principles of Boundary Formation in the Pregastrular Sea Urchin Embryo Ectoderm. The 2D oral grid of ectodermal (and future neurogenic) regulatory states is established in this embryonic region in the complete absence of cell migration. The regulatory states are imposed on the single-cell-thick ectodermal wall of the embryo, each cell inheriting from its parent the output of the immediately preceding spatial gene expression pattern. Careful attention to the temporal sequence of spatial expression of the regulatory genes constituting each boundary reveals some simple commonalities: the process is invariably asynchronous, and the boundaries are formed by mechanisms that depend directly on the order of regional gene expression. Therefore, the premise noted at the outset works: i.e., the first expressed gene in a given domain always executes a key role in formation of the eventual boundaries of the domain that this gene characterizes. The system therefore operates in a determinate way. Never do we encounter simultaneously expressed "bi-stable states" expressed within the same cells and mediated by dueling mutual repressors. However, once the regulatory state domains are formed, the canonical circuits enforcing them are exclusion circuits: the output of each regulatory state includes specific repressors of the regulatory state across the boundary. Each of the boundaries considered here illustrates these principles.

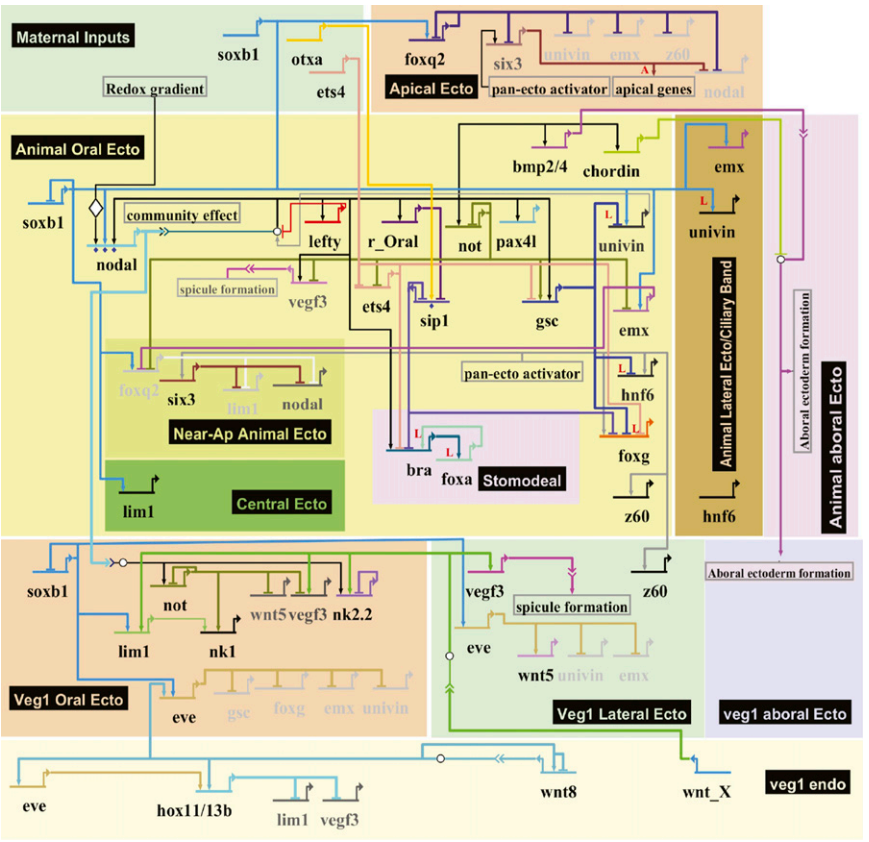
In the formation of the apical neurogenic/oral ectoderm boundary, the first player to be expressed is the *foxq2* repressor, responding to a maternal and zygotic pan-ectodermal activator, SoxB1. At the same time, *nodal* is expressed, driven by the same activator, by a redox-sensitive transcription factor that causes its expression to occur exclusively on the oral side of the embryo, and by feedback from its own signal transduction system (24, 35). One regulatory step later, Nodal signaling turns on *not*, which, after it is transcribed and its mRNA translated, represses *foxq2* in the oral ectoderm where it is expressed. The apical neurogenic/oral ectoderm boundary is formed with the aid of a second widely expressed *soxB1* target gene, the repressor *emx*, required in the near-apical oral ectoderm where *nodal* and *not* expression are weaker. These repressions confine *foxq2* expression to the apical domain. Then the exclusion functions kick in: *foxq2* represses *emx*, and *foxq2* plus *six3* repress *nodal* and consequently the whole oral ectoderm GRN within the apical domain. The neurogenic *foxq2* region is thus permanently segregated.

Within the oral ectoderm, another boundary forms—that separating the near-apical from the central oral ectoderm. Here another early regulatory player is *six3*, activated almost as early as is *foxq2* in the animal hemisphere, and by 18 h is expressed in the upper half thereof. The repressive target of *six3* plus *foxq2* is *lim1*, which because it also is driven by SoxB1 could express throughout the ectoderm, but because *six3* and *foxq2* are expressed first, *lim1* can be transcribed only up to the lower boundary of the *six3* expression domain, defining a central oral ectoderm region, whereas the *six3*-expressing region between the *foxq2* and *lim1* boundaries becomes the near-apical ectoderm. Thereafter, a further exclusion is instituted: *foxq2* excludes *six3* expression from the central apical plate and confines its expression domain to the surrounding near-apical ectoderm.

At the lower boundary of the ectoderm the regulatory state domains are formed in two successive steps. Here the first regulatory gene to be expressed in *veg1* is *eve*. Initially, several ectodermal regulatory gene expression domains overlap that of *eve*, but *eve* repression cancels their transcription, forming the boundary between *veg1* and the overlying oral ectodermal regulatory states. Later the first regulatory gene to be expressed in the portion of *veg1* to become endoderm is *hox11/13b*. Transcription of the ectodermal *lim1* regulatory gene is extinguished by *hox11/13b* repression, setting the boundary between *veg1* endoderm and *veg1* ectoderm, in which *lim1* continues to be expressed. The aboral/oral axial boundaries between the medial oral ectoderm and lateral ectodermal fates are also set sequentially: they depend on the build-up of the dominant Not repressor, which extinguishes transcription of a set of previously broadly expressed oral ectoderm genes.

To summarize, whereas a common activator is used to initiate the expression of most ectodermal genes, the complex patterns of gene expression are determined by the minuet of sequential repressions and thus by the encoded targeting of given genes by given repressors. These features cause the patterning process to be determinate and invariant.

Enhanced Territorial GRN Model. Fig. 4 incorporates the findings summarized here with our previously assembled GRN model for the oral ectodermal domain. However, the BioTapestry model accurately represents the spatial transactions within the oral ectoderm, as organized into the complex regulatory state domains shown in Fig. 1*A* for the 30-h embryo. The GRN model as portrayed indicates the linkages that are active or inactive in each domain. Over 60 regulatory linkages among about 30 transcription factor and signaling genes are included, the evidence for each of which is summarized in abbreviated form in Table S1. The Bio-



Additional data source for selected notes: L: T. Lepage Lab; A: Angerer's Lab

Fig. 4. The GRN model illustrating the genomic control of 2D expression pattern formation in the sea urchin ectoderm. This model is a BioTapestry presentation of all interactions among regulatory genes governing ectoderm regulatory state diversification up to the onset of gastrulation. The circuits show that domain-specific repressors are commonly used to define the boundaries along both embryonic axes. Evidence and references supporting the linkages shown in the network are summarized in Table S1.

Tapestry model presents the predicted topology of the oral ectoderm regulatory system, displaying its modular circuit features (4), such as double-negative gates, community effect circuits, exclusion circuits, feedbacks, etc. Space does not permit discussion of these individual features and the logic operations that they execute; suffice it to say, the pregastrular oral ectoderm GRN models will soon support a global logic analysis similar to that recently applied to the endomesoderm GRN model (7). Fig. 4 is incomplete in that it does not include the networks functioning within the aboral ectoderm, the lateral ectoderm, and the other ciliated band domains or the stomodeal domain, all to be presented elsewhere. However, Fig. 4 does encompass the network of regional cross-repressive exclusion functions and the repressions of repressor genes that underlie boundary formation in the oral ectoderm, the newly discovered outcome of this work.

Materials and Methods

Gene Cloning and Constructs. *hox11/13b* and *lim1* were previously described (11, 36). *univin*, *z60* (*egr*), *emx*, *eve*, and *foxq2* were PCR-cloned. The primer sets used for gene amplification are listed in Table S2. Gene models generated from sea urchin transcriptome analysis were used as a reference for primer design (37). cDNA prepared from various developmental stages was used as template for PCR. PCR products were purified and ligated into GEM-T EZ constructs. Cloned genes were PCR-amplified using the primer flanking the inert region, and PCR products were used to synthesize mRNA for microinjection or RNA probes for in situ.

Whole-Mount in Situ Hybridization. The protocol for whole-mount in situ hybridization (WMISH) to map gene expression has been described previously (38). Briefly, sea urchin embryos were fixed in glutaraldehyde solution. The fixed embryos were incubated in the hybridization buffer [50% (vol/vol) formamide, 5× SSC, 1× Denhardt's, 1 mg/mL yeast tRNA, 50 ng/mL heparin, and 0.1% tween-20] with 0.5 ng/μL digoxygenin- and fluorescein-labeled RNA probe(s) at 60 °C for 18 h. Posthybridization washes were hybridization buffer, 2× SSCT (2× SSC, 0.1% tween-20), 0.2× SSCT, and 0.1× SSCT, each 20 min at 60 °C. Subsequently, the antibody incubations were performed out at room temperature with 1:1,000 diluted anti-DIG Fab (Roche). The embryos were extensively washed before staining reaction, including six times with MABT buffer (0.1 M maleic acid, 0.15 M NaCl, and 0.1% tween-20), twice with AP buffer [100 mM Tris-Cl (pH 9.5), 100 mM NaCl, 50 mM $MgCl_2$, and 1 mM levamisole]. 5-Bromo-4-chloro-3-indolyl-phosphate (BCIP) and nitro blue tetrazolium were used for staining. In the double in situ hybridization, embryos were treated with glycine stop solution [0.1 M glycine (pH 2.2), 0.1% tween] after the first color reaction and then directly followed by the second antibody incubation [1:1,000 diluted anti-fluorescein antibody (Roche)]. 2-[4-Iodophenyl]-3-[4-nitrophenyl]-5-phenyl-tetrazolium chloride/BCIP were used to stain the embryo.

Microinjection and Expression Analysis. MASO sequences of *eve*, *foxQ2*, *six3*, and *hox11/13b* were previously described (5, 14, 15, 36). The *emx* MASO sequence was 5′-ATTGTCTCTTTTCAACCCTGTTTCT-3′. Concentrations of MASOs used for microinjection were 300 or 150 μm each in double MASO injection. Approximately 3 pL of MASO solution was injected into each fertilized sea urchin egg. The injection solution included 120 mM KCl. A total of 200 MASO-injected embryos were collected at different time points. RNA was prepared using Qiagen RNAeasy Micro Kit. Total RNA was reverse-transcribed with Bio-Rad iScript Kit.

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